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The adjuvant effect of hypertension upon diabetic peripheral neuropathy in experimental Type 2 diabetes

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ABSTRACT

Type 2 diabetes (DM) is the most common cause of peripheral neuropathy in the Western world. A comorbidity, hypertension, has been speculated to contribute to initiation or worsening of diabetic peripheral neuropathy. We studied adult rat models using genetic strains with DM (Zucker Diabetic Fat rats) ± hypertension (HTN (ZSF-1 rats)) to investigate the relative contributions of DM and HTN and the potential for additive effects of HTN upon existing DM for the development of peripheral neuropathy. Long duration sensorimotor behavioral and electrophysiological testing was complemented by histological and molecular methods. Only DM led to tactile and thermal hyperalgesia and affected motor nerve electrophysiology. Although DM led to marked loss of sensory amplitudes and to sensory conduction slowing, a mild additive effect from HTN contributed after 6 months of DM with worsening of slowing of sensory nerve conduction velocities, but without effect upon sensory amplitudes. At the sensory dominant sural nerve, mild (<10%) but greater degrees of myelin thinning were noted with DM and HTN combined, suggesting a mild additive effect. Matrix metalloproteinase (MMP) expression was increased only at the sural nerve in the presence of HTN with co-localization to Schwann cells and myelin. The effects of DM and HTN upon peripheral nerve are dissimilar, with HTN contributing to MMP upregulation at the sites of myelin thinning at sensory nerve fibers, potentially worsening comorbid DM. Together, our results indicate that HTN has a mild additive contribution to diabetic peripheral neuropathy at sensory peripheral nerve fibers manifesting with the loss of myelin thickness.

Introduction

The last decade has seen Type 2 DM (DM2) prevalence explode to become a troubling public health problem (Lipscombe and Hux, 2007; Ohinmaa et al., 2004; Schipf et al., 2012). Unlike type 1 DM, which typically begins at young ages and relates to autoimmune-induced insulin deficiency, DM2 usually begins in middle-later ages and is interrelated with insulin resistance. One of the most disabling complications of DM2 is the presence of diabetic peripheral neuropathy (DPN), a cause of peripheral sensory loss, incoordination, weakness, and pain. Although clearly related to hyperglycemic control (Tomlinson and Gardiner, 2008), other factors likely play a role in the initiation and progression of DPN. One potential factor is a frequent comorbidity of DM2: hypertension (HTN), which shares a number of systemic vascular complications. A prospective cohort study identified HTN as a vascular risk factor predicting incidence of DPN (Tesfaye et al., 2005). The mechanisms by which HTN may contribute to initiation or progression of DPN are unclear, but may relate to vascular hypoperfusion. HTN as a risk factor for peripheral neuropathy in the absence of DM2 is not well supported (Mold et al., 2004), and in some studies, HTN has a negative association with presence of peripheral neuropathy (Cho et al., 2006). It is possible that the effects of HTN only manifest in the presence of DM2; if so, its management may be crucial given the potential for HTN to manifest in the presence of DM2, even in children (Reinehr et al., 2008).

We examined animal models for DM2 (ZDF rat), along with the combination of DM2 and HTN in an obese hypertensive genetic strain of rat (ZSF-1) (Rafikova et al., 2008), as well as appropriate control rats. Furthermore, we speculated on the potential impact of matrix metalloproteinases upon peripheral nerve function in our rodent models of impaired glucose tolerance, hypertension and obesity (Shubayev et al., 2006). We found that the combination of DM2 and HTN gave a mild, late additive effect upon sensory nerve function and development of myelin thinning on top of DM2 alone.

Materials and methods

Animals

We used an established adult rodent model of DM2, the Zucker Diabetic Fat (ZDF) rat. A Zucker Diabetic Fat Lean (ZDF Lean) rat served as the control group for this cohort. The combination of DM2 and HTN was studied in a genetic hybrid, the ZSF-1 rat, formed by a cross

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between a ZDF female and a Spontaneously Hypertensive Heart Failure male rat (Rafikova et al., 2008). Serving as a control for the ZSF-1 rat was the ZSF-1 Lean rat (Rafikova et al., 2008). These combinations allowed us to study the impact of DM2 with and without HTN upon the peripheral nervous system, with a minimum of eight rats per cohort. All protocols were reviewed and approved by the University of Calgary Animal Care Committee using the Canadian Council of Animal Care and Principles of Animal Experiments (EU Directive 2010/63/EU) guidelines.

Whole blood fasting glucose measurements and weights were performed monthly with diabetes defined as previously described (Francis et al., 2009). Blood pressure was obtained by measurement at the tail artery monthly beginning at one month of age using an LE 5001 Pressure Meter (Panlab, Cornella Barcelona, Spain).

Behavioral testing

Behavioral testing to examine for sensory and motor manifestations was conducted every two weeks after establishing diagnoses of DM (or equivalent age) until endpoints. Training was initiated at one month of age — animals were acclimatized with placement into the appropriate apparatuses daily for one week, with a minimum of 1 h provided between sensorimotor tests.

We performed mechanical sensitivity and thermal hyperalgesia testing to determine sensory function. A Dynamic Plantar Aesthesiometer (Ugo-Basile, Milan) was used to obtain mechanical withdrawal thresholds. Rats were placed in clear acrylic boxes with a metal grid floor at 22 °C with acclimatization for 15 min before trials began. Stimuli consisted of application of a metal filament (0.5 mm) applying a linearly increasing force ramp (2.5 g/s) to the middle of the hindpaw plantar surface. A maximal threshold of 50 g was imposed to limit tissue damage. The paw withdrawal threshold (PWT) was averaged from three consecutive tests and the mean value was used for analyses; there were five minute intervals between each test. Mechanical allodynia was defined as a reduction in threshold as compared to the baseline PWT.

We performed thermal hyperalgesia in the same acrylic clear boxes under the same conditions but on top of a glass floor. A mobile radiant heat source (Hargreaves apparatus) placed under the glass floor focused heat onto the middle of a single hindpaw; the latency (seconds) to paw withdrawal was measured. The heating rate consistently increased from 30 °C to 58 °C over a maximum of 60 s (to prevent hindpaw injury) for each test. Before and after thermal testing, paws were inspected to ensure absence of thermal damage. The withdrawal latency of both hindpaws from three consecutive trials was averaged, and the mean value was used.

Dynamometry and Rotarod testing were performed for motor assessment. Dynamometry (grip strength testing) utilized a Chatillon DFIS-2 digital force measurement dynamometer (Ametek, Inc., Paoli, PA) using a sampling rate of 1 kHz to measure peak values. Rats were held at the posterior cervical region with hind limbs free while one hind limb was placed on the pull rod encircled by digits. For combined forelimb testing, both forelimbs were placed at the pull rod with all bilateral digits encircling the rod. After seven daily trials beginning at one month of age, we began to capture biweekly data for peak strength (g) for each hind limb and for the combined forelimbs with the three highest forces for five consecutive trials recorded.

The Rotarod test (Microprocessor Controlled Rota-Rod Treadmill for Rats, Model 57602, Ugo Basile, Italy) was used for locomotor testing. Acclimatization was performed over seven daily training trials at a slow speed of 10–15 rpm with walking on a revolving drum beginning at one month of age. Testing commenced using a graded protocol immediately following this using a maximum threshold of 150 s for trial, with latencies to falling off of the Rotarod as endpoint. We obtained an average from three trials every two weeks for each rat, with five–ten minute latencies between trials.

Electrophysiological testing

We performed electrophysiological assessment of sciatic nerve function as described previously (Francis et al., 2009) using isoflurane anesthesia. At least eight rats in each intervention group underwent monthly electrophysiological testing beginning at one month of age until six months of DM had occurred. Sural nerve stimulation at the lateral Achilles tendon using platinum subdermal stimulation needle electrodes (Grass Instruments, Astro-Med, West Warwick, RI) for orthodromic sensory conduction studies was performed. Receiving electrodes placed at the sciatic notch permitted measures of the sensory nerve action potential (SNAP) amplitude and sensory nerve conduction velocity (SNCV). Motor studies used stimulating electrodes placed at the sciatic notch or popliteal fossa for sciatic nerve stimulation, with receiving electrodes placed at the abductor pollicis brevis muscle for calculation of compound motor nerve action potential (CMAP) baseline-peak amplitude and motor nerve conduction velocity (MNCV). A heating lamp was used to maintain near-nerve temperature constant during testing at 37 ± 0.5 °C.

We performed an incremental method to obtain motor unit number estimation (MUNE) (31). We increased stimulus intensity slowly from subthreshold levels until a small, all-or-nothing response was evoked (initial SMUP amplitude) appearing with a consistent amplitude and appearance on three separate occasions in the absence of smaller evoked amplitude responses. Stimulation intensity was slowly increased to permit visualization of stepwise quantal increments in amplitude, repeated for a minimum of 25 increments, unless not obtainable. Individual motor unit amplitudes were determined by subtraction of amplitudes of each response from the previous response. An average of individual values was used to estimate the average SMUP amplitude; any individual values differing by two SDs greater than or less than the average SMUP were removed. We divided the resulting average SMUP into the supramaximal CMAP amplitude response to yield the MUNE.

Tissue harvesting

After six months of DM2, rat weight and blood glucose final measurements were performed. Euthanasia was induced using intraperitoneal pentobarbital (60 mg/kg); intracardiac blood was obtained for HbA1C measurements and for plasma for insulin measurements first. Insulin plasma concentrations were measured using microparticle enzyme immunoassays (MEIA) (MEIA Insulin, IMX System, Abbott Laboratories) which have a minimal detection amount of 0.1 pmol/L. Then, surgical collection of tissues was performed for the sural and sciatic nerves, lumbar dorsal root ganglia (DRG), and footpads. Left-sided tissues obtained were placed in cacodylate buffered glutaraldehyde, then cacodylate buffer for later Epon embedding (Epon 812 resin, Canemco Inc., Canada) for morphometric studies. Right sided tissues were immediately fresh frozen at −80 °C and stored at −80 °C for protein analyses.

Quantitative morphometry of peripheral nerve and dorsal root ganglia

For peripheral nerve and dorsal root ganglia (DRG), samples were embedded in Epon for ultramicrotome sectioning at 1 μm and staining with 0.5% toluidine blue (Toth et al., 2008). A single observer blinded to cohort allocation performed image analysis (Zeiss Axioskope at 400× and 1000× magnification) to measure the number, axonal area, and myelin thickness for all myelinated fibers for each of twenty-five non-adjacent transverse nerve sections. For DRGs, only those neurons with visible nuclei were counted within a pre-sized area using 25 non-adjacent sections with separation of approximately 300 μm (in order to avoid double counting) for each L4-6 DRG to calculate neuronal density. Differentiation of satellite and Schwann cells from neurons was based upon the presence of Nissl substance and size. All measurements